

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ :
C12P 21/00, C07K 15/00
C12N 5/00, 15/00, G01N 33/577
// (C12P 21/00, C12R 1:91)

A2

(11) International Publication Number: WO 87/ 07302

(43) International Publication Date: 3 December 1987 (03.12.87)

(21) International Application Number: PCT/EP87/00264

(22) International Filing Date: 20 May 1987 (20.05.87)

(31) Priority Application Number: 86/07583

(32) Priority Date: 27 May 1986 (27.05.86)

(33) Priority Country: FR

(71) Applicant (for all designated States except US): LABOR-
ATOIRES UNICET [FR/FR]; 92, rue Baudin, F-
92307 Levallois-Perret (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only) : BONNEFOY, Jean,
Yves [FR/FR]; Les Terrasses de Parsonges, Bâtiment
E, F-69570 Dardilly (FR). WIJDENES, John [NL/
FR]; 8, rue Jean Perréal, F-69008 Lyon (FR). PER-
ONNE, Catherine [FR/FR]; 70, rue de la Charité, F-
69002 Lyon (FR). AUBRY-LACHAINAYE, Jean-Pi-
erre [FR/FR]; 4, rue des Fraisières, F-69630 Chaponost
(FR). BANCHEREAU, Jacques [FR/FR]; 25, avenue
Paul Santy, F-69130 Ecully (FR).

(74) Agent: DURAND, Yves; Cabinet Weinstein, 20, ave-
nue de Friedland, F-75008 Paris (FR).

(81) Designated States: AT (European patent), BE (Euro-
pean patent), CH (European patent), DE (European
patent), FR (European patent), GB (European pa-
tent), IT (European patent), JP, LU (European pa-
tent), NL (European patent), SE (European patent),
US.

Published

Without international search report and to be repu-
blished upon receipt of that report.

(54) Title: MONOCLONAL ANTIBODIES TO HUMAN LYMPHOCYTE IgE RECEPTORS, HYBRIDOMAS PROD-
UCING SUCH ANTIBODIES, AND KITS FOR USING SUCH ANTIBODIES

(57) Abstract

Monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes, to monoclonal antibodies to hu-
man lymphocyte IgE receptors, and to hybridomas producing such antibodies. These antibodies are preferably of the im-
munoglobulin subclass IgG₁. They can be used in assaying soluble Fc_εR_{II} and cells carrying these Fc_εR_{II} receptors. The in-
vention further provides kits for assaying soluble Fc_εR_{II} and also cells carrying these Fc_εR_{II} receptors by means of
monoclonal antibodies described herein.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

MONOCLONAL ANTIBODIES TO HUMAN LYMPHOCYTE IgE RECEPTORS,
HYBRIDOMAS PRODUCING SUCH ANTIBODIES,
AND KITS FOR USING SUCH ANTIBODIES

I. INTRODUCTION

This invention relates to novel monoclonal antibodies to the human lymphocyte IgE receptors, that is, to monoclonal antibodies directed at the receptor for the Fc fragment of human IgE which is present in lymphoid cells ($FC_\epsilon R_{II}$), to hybridomas producing such antibodies, to kits for using such antibodies, e.g. in assaying or detecting these receptors on cells, and to related soluble molecular entities of the IgE binding factor type (=IgE BF).

It is well known in the art that it is possible to obtain a cell line which is able to produce a homogenous, i.e. monoclonal, antibody. The basic technique (Kohler and Milstein, Nature 256, 1975) comprises the fusion of mouse myeloma cells with spleen cells to form hybridoma cells and selection from these of clones capable of producing the desired antibody. This general procedure has also been described in U.S. Patents 4,364,932, 4,364,934, 4,364,935, 4,364,937 and 4,361,550.

Although the general method has been known for some years, the preparation and selection of each suitable hybridoma presents its own special

difficulties. There is indeed no certainty that a suitable hybridoma will be found and, equally, there is no certainty that the hybridoma will produce an antibody having the desired properties.

5 Monoclonal antibodies have a variety of uses, in particular for the isolation and purification of the proteins to which they are specific or for assaying them e.g. in a diagnostic kit; see for example PCT published applications WO81/02899 and WO82/01773.

10 The receptor for the Fc fragment of Immunoglobulin E ($\text{Fc}_\epsilon\text{RII}$) is a glycoprotein expressed on the surface of certain human cells. It has the capacity of binding IgE and therefore can be considered an IgE binding factor. Suppressive IgE BFs would be of
15 considerable interest in the therapy of diseases linked to elevated IgE levels, such as allergic rhinitis, atopic dermatitis and asthma. IgE potentiating factors, on the other hand, would have utility in the treatment of conditions where increased IgE levels might be required
20 (e.g., elimination of parasites). Assays for such factors exist, but the availability of monoclonal antibodies (Mabs) would greatly help in designing highly sensitive and specific immunochemical assays.

It is therefore an object of the present
25 invention to provide the following:

Monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes;
Monoclonal antibodies to the human lymphocyte IgE receptors;
30 Methods for their preparation;
Hybridomas producing them;
Assays using them (for detecting them on human cells);
Kits using them for detection of soluble forms
35 (e.g., in body fluids or culture supernatants).

The invention therefore provides monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes. The invention further provides monoclonal antibodies to the human lymphocyte IgE receptors. These monoclonal antibodies are preferably of the immunoglobulin subclass IgG₁. The invention further provides monoclonal hybridomas capable of producing these monoclonal antibodies.

According to the invention, monoclonal antibodies inhibit the binding of soluble IgE to human lymphocytes, and are identical or similar to monoclonal antibodies produced from hybridomas obtained according to the following successive steps :

a) The culture of hybridomas is obtained, using several separate containers, by fusion of mice myeloma cells with spleen cells obtained from mice immunized by injections of cells which strongly express IgE receptor, e.g. RPMI 8866, preferably in a medium eliminating unfused myeloma cells and unfused spleen cells, e.g. in a HA medium;

b) the supernatants of the hybridoma cells of each container are tested for the presence of antibodies inhibiting the binding of IgE to the said cells;

c) hybridomas producing antibodies inhibiting the binding of IgE to the said cells are selected;

d) these selected hybridomas are cloned, preferably by the limiting dilution technique, these clones being chosen so as to obtain the desired monoclonal antibodies by culturing the hybridomas.

These monoclonal antibodies are preferably of the immunoglobulin subclass IgG₁.

The aforesaid antibodies are the monoclonal antibody Mab 25 produced by the hybridoma 9P25 A 3/13

deposited at the Institut Pasteur under N° I-548, and the monoclonal antibody Mab 135 produced by the hybridoma 9P 135 D 6/5 deposited at the Institut Pasteur under N° I-549.

5 The assays for soluble IgE BF's can be of two types, a competition assay or a double sandwich assay (which requires two Mabs binding to different epitopes of the relevant antigen).

10 In the competition assay, a human IgE binding factor (IgE BF) labelled e.g. with an enzyme (for example E. coli β -galactosidase) or a radioisotope competes with a non-labelled one (present in the sample to be assayed or a standard) for binding to a Mab linked to a solid support. This requires only one Mab.

15 In order to produce Mabs specific for human IgE binding factors, mice were immunized with cells expressing on their surface such molecules at high density. Alternatively, mice could be immunized with soluble IgE binding factors.

20

II. ASSAY FOR HUMAN $Fc\epsilon R_{II}$ AND CHOICE OF THE CELL LINE EXPRESSING $Fc\epsilon R_{II}$ AT HIGH DENSITY.

INTRODUCTION

25

30 The human cell line RPMI 8866 is known to express $Fc\epsilon R_{II}$ at relatively high density. An assay has been established which permits detection of $Fc\epsilon R_{II}$. Briefly, cells to be assayed are successively incubated with IgE, a monoclonal (or polyclonal) anti-IgE antibody, and fluorescent microspheres coupled with anti-mouse (or rabbit) immunoglobulin antibody. The percentage of cells

binding to the fluorescent microspheres. (corresponding to the percentage of cells expressing $FC_{\epsilon}R_{II}$) is determined by flow cytometry. Details of this are given in a separate section.

5 This assay was found suitable for the detection of human IgE BF: Preincubation of IgE with IgE BF results in a decreased binding of IgE to its receptor so that a decreased percentage of cells bind to the fluorescent microspheres. This work demonstrated that
10 RPMI 8866 was the best cell line for immunizing mice since it carried much $FC_{\epsilon}R_{II}$.

Mabs against $FC_{\epsilon}R_{II}$ can be screened for their ability to inhibit the binding of IgE to RPMI 8866 cells. Cells are first incubated with the Mab to be
15 assayed and then IgE is added. The other steps are carried out as described above.

FLOW CYTOMETRIC METHOD FOR DETECTION OF FC_{ϵ} RECEPTORS AND IgE BINDING FACTORS USING FLUORESCENT MICROSPHERES

20

MATERIALS AND METHODS

Antibodies and proteins:

Human myelomatous IgE was purified by ion
25 exchange chromatography (DEAE 52) using a NaCl gradient (0.01M - 0.3M, pH 7.2). According to SDS-PAGE and silver staining, the IgE was pure and uncontaminated by other proteins.

To detect IgE, a monoclonal anti-IgE (Hybritech
30 Inc., La Jolla, CA) and a polyclonal anti-IgE were used. The polyclonal antiserum was prepared by immunizing rabbits with purified IgE PS, and the antiserum was rendered monospecific for IgE by passage over IgG, IgA and IgM immunoabsorbant columns (Affigel-10
35 Biorad, Richmond, CA).

A rabbit anti-mouse Ig antiserum and a goat anti-rabbit Ig antiserum have been prepared in our laboratory. Their IgG fractions were isolated by column chromatography using Trisacryl GF 05 and DEAE Trisacryl (Industrie Biologique Francaise, Genevilliers, France). Human IgG, IgA and IgM were purified from myeloma or Waldenstrom sera using ion exchange chromatography according to standard procedures.

10 CELLS:

WIL2WT (an EBV (= Epstein-Barr Virus) lymphoblastoid cell line), Daudi (a Burkitt lymphoma), Molt 4 (a T leukemia), HL60 (a promyelomonocytic leukemia) and U937 (a promonocytic cell line) were obtained from the ATCC (American Type Culture Collection; Rockville, Maryland). RPMI 8866 (an EBV lymphoblastoid cell line) was kindly provided by Dr. K. Ishizaka. UD38 is an EBV lymphoblastoid cell line which has been established in our laboratory by Dr. F. Rousset after infection of normal peripheral blood B cells with EBV. All cells were cultured in RPMI 1640 (Flow, Irvine, Scotland) supplemented with 10% heat inactivated fetal calf serum (Flow), 2mM glutamine (Flow), 100 U per ml penicillin and 100 µg per ml streptomycin (Flow).

25

Coupling of proteins to microspheres

0.1 mg of the protein to be coupled is dissolved in 1 ml of a solution of EDAC (N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride) (0.1 mg per ml distilled water).

30

100 µl sonicated (1 min in a Branson 321) microspheres (Fluoresbrite carboxylate diameter 0.57 µ, Polyscience, Warrington, PA, ref 15700) are added. The suspension is incubated overnight at 4°C on a rocking table. After this incubation, the microspheres are

35

centrifuged (10000 g, 5 min) and washed three times with PBS-BSA (phosphate-buffered saline containing 1% bovine serum albumin). The microspheres are finally kept at 4°C in PBS-BSA supplemented with 15 mM sodium azide. The
5 suspension is sonicated for 1 minute before each experiment.

Staining protocol

Cells are adjusted to 10^7 /ml in PBS-BSA. 5 x
10 10^5 cells (50 µl) are distributed per well of 96 conical wells microtiter plates (Flow, Reference number: 7632105.)

The cells are first incubated 45 min with monomeric IgE (0.5 to 2 µg per 5×10^5 cells). Cells are
15 then washed with PBS-BSA. A polyclonal anti-IgE antibody appropriately diluted (e.g., 1/2000) is added. After 45 min, the cells are washed with PBS-BSA. The anti-rabbit Ig microspheres (dilution 1/50) are added.

This cell suspension is layered on top of 3 ml
20 heat-inactivated and filtered FCS in conical tubes (Falcon Ref 2099). After centrifugation at 100 X g for 15 min, the supernatant is slowly aspirated and PBS-BSA (150 µl/well) is added to the cell samples which, after resuspension, are analysed by flow cytometry. All steps
25 are carried out at 4°C.

Fluorescence analysis

Fluorescence analysis was performed with a flow
30 cytometer, FACS 440 (Becton Dickinson, Sunnyvale, CA), equipped with a 5 W argon laser running at 488 nm, 0.5 W. Fluorescence parameters were collected using the built-in logarithmic amplifier after gating on the combination of forward light scatter (FLS) and
35 perpendicular light scatter (PLS) which was used to distinguish viable cells from non-viable ones.

Data were stored in list mode and analysed with a PDP 11/23.

III. IMMUNIZATION OF MICE AND PRODUCTION OF HYBRIDOMAS
5 SECRETING FC_γR_{II}-SPECIFIC MABS

1. Mice are immunized three times with 50×10^6 RPMI 8866 cells injected intraperitoneally in phosphate-buffered saline (PBS). The type of mouse used should not be critical but good results are achieved with BALB/c
10 females. The number of injections and the quantity of cells administered must be such that useful quantities of suitably primed splenocytes are produced. The serum of properly immunized mice inhibits the binding of IgE to RPMI 8866 cells.

15

2. The immunized mice are killed, their spleens are removed and spleen suspensions are prepared. This procedure follows well-known techniques.

20 3. The spleen cells are fused with mouse myeloma cells. The technique for fusing myeloma cells with spleen cells is well known. Most preferably the fusion is achieved by warming a mixture of the two cell types with an appropriate fusion promoter, e.g. polyethylene-
25 glycol (PEG) having an average molecular weight from about 1000 to about 4000 (PEG1000). Several mouse myeloma cell lines are known and easily available. Preferred are cell lines which are HGPRT-deficient (HGPRT = Hypoxanthine Guanosyl Phosphoribosyl Transferase) and
30 accordingly will not survive in HA (culture medium comprising hypoxanthine and azaserine). Preferably the myeloma cell line used should be of the non-secreting type in that it does not itself produce any antibody. A suitable cell line for the purpose of this invention is
35 the so-called NS1 cell line. These cells were derived from P3/X63-A8 myeloma cells by Kohler and Milstein.

4. The fused spleen cells are cultured in several separate containers (e.g. in 24-well plates) according to standard procedures. The cell cultures obtained in step 3 are mixtures of fused spleen cells (hybridoma cells), unfused spleen cells and unfused myeloma cells. Preferably the cultivation is carried out in a medium which will eliminate the unfused myeloma cell line, e.g. in an HA medium. Those unfused spleen cells which are non-malignant will normally die after a short period of time, whereas the fused cells, which are HGPRT⁺, can grow in HA medium.
5. The supernatants of the hybridoma cells in each container are tested for the presence of anti-Fc_εR_{II} antibodies. The hybridomas are screened for their ability to inhibit the binding of IgE to RPMI 8866 cells. This test may conveniently be carried out by means of the bead assay described above.
6. Hybridomas producing the desired antibodies are selected and then are cloned preferably by the limiting dilution technique.
7. The desired antibodies are produced by means of the selected hybridomas. This production may be achieved in vitro by culturing the hybridoma in a suitable medium followed by isolation of the antibody, but this method may not yield sufficient quantities. For producing larger quantities of the antibody, an in vivo method is preferably used. The hybridoma is injected back into mice where it will cause production of ascites fluid containing substantial quantities of the desired antibody, which is then isolated according to standard procedures.

IV. CHARACTERISTICS OF THE PRODUCED HYBRIDOMAS1. Inhibition of IgE binding

Two hybridomas, 9P25 and 9P135 D6/5, which gave two clones 25 and 135 D6/5 respectively, have been selected since they produce Mabs (called Mab 25 and Mab 135 respectively) that inhibit the binding of IgE to RPMI 8866 cells; see Table I:

TABLE I

Inhibition of IgE Binding by Mab 25 and Mab 135
Antibodies on the RPMI 8866 Cell Line

c-	c+	Mab 25	Mab 135
8	82	29 (71.6)	56.3 (34.7)

Values are given as percentages. Unbracketed figure gives percentage of positive cells and bracketed figure gives percentage inhibition.

Key:

c-: negative control obtained by incubation of RPMI 8866 cells with polyclonal anti-IgE antibody (1/1000) and anti-rabbit Ig-coated microspheres.

c+: positive control obtained by incubation of RPMI 8866 cells with 1 μ g IgE, polyclonal anti-IgE antibody and anti-rabbit Ig-coated microspheres.

Hybridomas 9P25 and 9P135 D6/5 have been deposited at the Institute Pasteur, Paris, France under the numbers I 568 and I 549 respectively.

5 2. Binding pattern to cell lines

a) Technical aspects

Cells lines are maintained in the standard culture medium RPMI 1640 described above.

10 Cytofluorographic analysis of monoclonal antibodies binding to cell lines was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse immunoglobulins (G/M FITC) (Grub Laboratories, Vienna, Austria) utilizing a FACS 440. In brief, 5×10^5 cells were treated with 0.15 ml of the Mab
15 (diluted or undiluted), incubated at 4°C for 30 minutes, and washed twice. The cells were then reacted with 0.15 ml of a 1:40 dilution G/M FITC at 4°C for 30 minutes, centrifuged, and washed three times. Cells were then
20 analyzed on the FACS 440, and the intensity of fluorescence per cell was recorded on a pulse-height analyzer. Background staining was obtained by substituting a 0.15 ml aliquot of 1:500 ascites from a mouse intraperitoneally injected with a non-producing
25 hybrid clone.

b) Mab 25 (Table II)

It does not bind to Molt 4 cells (a T cell leukemic line); Daudi (a Burkitt lymphoma) and a wide
30 number of different IL₂-dependent human T cell clones (although some of them are found weakly positive). It binds to EBV infected lymphoblastoid B cell lines. It binds weakly to the myelomonocytic cell line U937. The binding of this antibody to the cell lines correlates very well with their Fc_γR_{II} expression.

TABLE II

Phenotypic expression of Mabs anti $Fc_\epsilon R$ on
different human cell lines

5

		* $Fc_\epsilon R_{II}$	Mab 25	Mab 135	**Mab Anti-DR	Control
10	U 937	±	16.4	13.4	95.0	1.4
	Molt 4	-	0.8	0.6	1.2	0.2
	DAUDI	-	2.1	96.4	99.2	0.9
15	RPMI 8866	+	94.1	91.8	99.8	1.1
	MO 14	+	97.8	98.0	98.3	4.8
20	BME	+	97.9	89.5	99.5	10.4

*Determined by microsphere (bead) assay described above.

**Clone L243 obtained from Becton Dickinson. DR antigens are histocompatibility antigens of Class II.

25 c) Mab 135 (Table II).

It does not bind to Molt 4 cells but does bind to Daudi cells as well as to all the IL_2 -dependent $Fc_\epsilon R_{II}$ -negative T cell clones that have been tested. It also binds to all the EBV infected lymphoblastoid cell lines which have been tested and to the EBV negative B cell lines MO14 and BME. It binds weakly to U937.

In summary, Mab 135 is able to prevent binding of IgE to $Fc_\epsilon R_{II}$ positive cells; and it also recognizes antigenic determinants which are present on $Fc_\epsilon R_{II}$ negative cells.

3. Binding pattern to human blood, tonsil and spleen cells

a) Technical aspects

Blood, tonsil and spleen mononuclear cells are isolated according to standard procedures. Tonsils were
5 obtained at tonsillectomy from children with chronic tonsillitis and spleens were obtained from operative specimens of trauma patients. Spleens and tonsils were made into single cell suspensions (in PBS) by being pressed through wire mesh. These cell suspensions as
10 well as diluted blood (in PBS) were next layered over Ficoll-Hypaque and centrifuged (following the technique of Boyum, Scand. J. Clin. Lab. Invest. 21 (supp. 97): 77, 1968). Mononuclear cells were harvested from the Ficoll interface and washed with PBS.

15 Cytofluorographic analysis of monoclonal antibodies with all cell populations was performed by indirect immunofluorescence as described earlier. Double fluorescence analysis was also performed in order to determine to which cell subpopulation the Mabs bind. The
20 experimental protocol is described in Table III.

Data are summarized in Tables IV, V and VI.

TABLE IIIPROCEDURE FOR DOUBLE STAINING CELLS WITH
TWO MONOCLONAL ANTIBODIES

- 5 1. Adjust cells at 10^7 /ml in PBS, 1% BSA, 0.01% Azide.
2. Add the first monoclonal antibody at the appropriate concentration to 40 μ l of the cell
10 suspension (in microtiter tray wells).
3. Incubate 15 minutes at 4°C.
- 15 4. Wash twice with PBS, 1% BSA, 0.01% Azide.
5. Resuspend cells in 100 μ l of a 1/150 dilution in PBS, FITC conjugated F(ab')₂ fragments goat anti-mouse immunoglobulins (Grub, Vienna, Austria).
20
6. Incubate 15 to 30 minutes at 4°C.
7. Wash twice in PBS-BSA-Azide.
- 25 8. Resuspend cells in 100 μ l PBS-BSA containing 1% mouse serum.
9. Incubate 20 minutes at 4°C and wash twice.
- 30 10. Resuspend cells in 40 μ l of PBS-BSA.
11. Add the second monoclonal antibody conjugated with biotin.
- 35 12. Incubate 15 minutes at 4°C.

13. Wash twice with PBS-BSA-Azide.
14. Resuspend cells in 40 μ l PBS-BSA-Azide and add
Avidin conjugated to Phycoerythrin (Becton
Dickinson).
15. Wash twice and resuspend cells in 250 μ l PBS-BSA.
16. Analyze with FACS.

In Tables IV - VI:

- indicates negatively reacting cells (Mab unbound);

5 + indicates positively reacting cells (Mab fixed).

Leu 1 is a Mab commercialized by Becton Dickinson which specifically binds to T cells.

10 B1 is a Mab commercialized by Coulter Immunology which specifically binds to B cells.

Anti-DR is the Mab defined in Table II.

15 Leu M3 is a Mab commercialized by Becton Dickinson which specifically binds to monocytes.

TABLE IVPERCENTAGE OF SPLEEN CELLS BOUND BY MABS

5	SPLEEN*		Mab 25		Mab 135	
			-	+	-	+
	TOTAL CELLS		4.8		52.2	
10						
		-	67.3	4.8	15.3	48.3
	Leu 1	(31.6)				
		+	27.9	<1	36.4	<1
15						
		-	39.2	<1	47.4	10.1
	B1	(54.8)				
20		+	56.4	4.4	3.0	39.5
		-	36.5	<1	40.7	<1
	Anti-DR	(61.3)				
25		+	58.3	5.0	1.7	57.6
		-	90.0	4.5	47.6	47.8
30	Leu M3	(5.6)				
		+	4.4	1.1	1.3	3.3
35	* Control:non-related Mab: 0.7					

TABLE V

PERCENTAGE OF TONSIL CELLS BOUND BY MABS

5	TONSILS*		Mab 25		Mab 135	
			-	+	-	+
	TOTAL CELLS		9.4		33.2	
10						
		-	53.4	9.4	26.2	27.6
	Leu 1 (40.3)					
		+	37.2	<1	46.2	<1
15						
		-	50.1	4.4	60.4	9.7
	B1 (41.6)					
20		+	39.2	6.3	3.1	26.8
		-	50.7	<1	62.3	<1
	Anti-DR (43.8)					
25		+	41.8	7.5	2.5	35.2
		-	90.5	7.6	65.1	33.9
30	Leu M3 (2.0)					
		+	<1	1.9	<1	1.0
35	* Control:non-related Mab: 0.9					

TABLE VI

PERCENTAGE OF PERIPHERAL BLOOD MONONUCLEAR CELLS
BOUND BY MABS

5	PBL*		Mab 25		Mab 135		
			-	+	-	+	
10	TOTAL CELLS		4.0		7.3		
	Leu 1	(67.5)	-	28.6	5.7	28.1	7.3
15			+	65.7	<1	64.6	<1
	B1	(8.1)	-	90.3	<1	87.6	5.1
20			+	6.6	3.1	1.6	5.7
	Anti-DR	(20.1)	-	76.5	<1	78.5	<1
25			+	19.9	3.6	13.4	8.1
	Leu M3	(12.0)	-	84.9	4.3	80.1	8.2
30			+	9.7	1.1	9.5	2.2
35	* Control:non-related Mab: 0.7						

b) Mab 25

This antibody binds to approximately 4% of blood mononuclear cells, 4.8% spleen mononuclear cells and 9.4% tonsil mononuclear cells. It does not bind to T cells (Leu 1 positive). It preferentially binds to B cells (B1 positive) but not all of them. It also stains some non-T non-B lymphocytes, some Leu M3⁺ cells (specific for monocytes) and some Leu M3⁻ cells.

10 c) Mab 135

This antibody binds to approximately 7.3% blood mononuclear cells, 52.2% spleen mononuclear cells and 33.2% tonsil mononuclear cells. It does not bind to T cells (Leu 1 positive). It binds to almost all B cells (B1 positive) and to a fraction of the monocytes (Leu M3 positive). It binds to almost all the DR positive spleen and tonsil mononuclear cells but only to a fraction of blood DR positive cells.

20 4. Binding pattern to activated PBL

a) Technical aspects

Isolated peripheral blood mononuclear cells are resuspended at 5×10^5 cells/ml into culture medium (RPMI 1640 + glutamine 2 mM + penicillin (100 U/ml) + streptomycin (100 µg/ml) + 10% Fetal Calf Serum) in the presence of the following mitogens:

Phytohemagglutinin 1% (PHA) (GIBCO, Grand Island); Concanavalin A 10 µg/ml (Con A) (Pharmacia, Uppsala, Sweden); Staphylococcus aureus strain Cowan I, 0.01% (SAC) (Pansorbin, Calbiochem, La Jolla, Ca.); Pokeweed Mitogen, 1/100 dilution (PWM) (GIBCO, Grand Island, NY). Cells were harvested five days later, washed three times and stained with the Mabs as described earlier. Data are given in Table VII.

TABLE VII

BINDING OF MABS 25 AND 135 TO PBL
(PERIPHERAL BLOOD MONONUCLEAR CELLS)
STIMULATED FOR 5 DAYS WITH VARIOUS MITOGENS

		CONTROLS	MAB 25	MAB 135	
10	CON A	3.6%	2.3%	42.8%	EXPT 1
	PHA	5.9%	5.9%	49.3%	
	PWM	1.1%	4.2%	28.8%	
	COWAN	7.7%	21.0%	23.8%	
15	CON A	ND	ND	ND	EXPT 2
	PHA	4.7%	16.4%	52.2%	
	PWM	0.8%	1.4%	42.5%	
20	COWAN	4.1%	3.2%	20.5%	

b) Mab 25

Its expression is only slightly or very slightly increased upon mitogenic activation.

c) Mab 135

The percentage of cells reacting with Mab 135 is increased upon mitogenic stimulation.

5. Immunoprecipitation analysis

a) Technical aspects

a1) Labelling of cells:

(50 to 100) $\times 10^6$ cells are first washed twice with PBS and then resuspended in 2 ml PBS. To this

suspension the following reagents are added: 0.5-1 mCi Na^{125}I (Amersham,) 100 μl lactoperoxidase at 5 mg per ml (Calbiochem) and 50 μl H_2O_2 (0.03% in water). The suspension is agitated for 3 minutes at room temperature. Another 100 μl lactoperoxidase and 50 μl H_2O_2 are added. The suspension is again agitated for 3 minutes at room temperature. This step is repeated once again and the cells are finally washed with PBS containing 5mM KI. The labelled cells are then resuspended at 5×10^6 per ml in PBS containing 0.5% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.01 M benzamidine hydrochloride, 0.05M aminocaproic acid, 20mM iodoacetamide, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin and 100 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, and are incubated at 0°C for 20 minutes. The cells are centrifuged at 3000X g for 20 minutes and the supernatant is stored at -70°C . The lysate is clarified by centrifugation at 100,000X g for 30 minutes.

20 a2) Immunoprecipitation of membrane antigens:

The lysates are first precleared overnight with an ascite containing a non-related monoclonal antibody. Then, a rabbit anti-mouse immunoglobulin coupled to formalinized Staphylococcus aureus strain Cowan is added. After centrifugation the lysate is ready for the immunoprecipitation, which is performed as in the preclearing step except that the non-related Mab is replaced by the antibody to be assayed.

30 b) Mab 25:

It specifically precipitates a 42-44 Kd molecule which corresponds to the established data for $\text{Fc}_\gamma\text{RII}$ using affinity chromatography on immobilized IgE.

c) Mab 135:

It immunoprecipitates three different molecular entities: a 44-46 Kd molecule (which may be different from that precipitated by Mab 25) and also molecules of about 35 Kd and about 30 Kd.

6) Determination of the affinity (K_a) of Mab 25

The affinity of Mab 25 for its specific cell surface antigen was performed according to Trucco and de Petris (Immunological Methods, Vol III, Ed: I. Lefkovits, B. Pernis, Academic press, 1981, pp. 1-26).

a. Technical aspect

al. Iodination of Mab 25

50 μ g of Mab 25 in 100 μ l of PBS are mixed with 1 mCi Na^{125}I (5 μ l) and chloramine T (5 μ l at 0.8 mg per ml in PBS). After a 10 minutes' incubation period at room temperature under gentle shaking, 5 μ l sodium bisulphite (1 mg/ml in PBS) and 250 μ l of ovalbumin (5% in PBS) are added to stop the reaction. The solution is passed over a PD10 column (Pharmacia, Sweden) pre-equilibrated with PBS containing 0.1% Tween 20 (Merck, Germany) and 1% bovine serum albumin. This step permits recovery of the iodinated antibody without free iodine.

a2. Determination of the affinity and of the number of receptors

Increasing concentrations (0.1 μ g to 4 μ g) of the iodinated Mab 25 are added to 10^6 RPMI 8866 cells in a final volume of 1 ml of RPMI 1640 medium containing 2% BSA. For antibody concentrations of 0.3, 0.5 and 1 μ g/ml,

additional tubes are prepared in which non-labelled Mab 25 is added in a 500-fold excess in order to determine non-specific antibody binding. Binding lasts 3 hours at 4°C. Cells are finally washed three times and the
5 radioactivity in the final cell pellet is determined in a gamma counter. The data are finally plotted using the standard Scatchard analysis.

10 b. Affinity and number of receptors of Mab 25

The affinity of Mab 25 for the RPMI 8866 $FC_\epsilon R_{II}$ is $1.2 \times 10^8 M^{-1}$ and 435,000 receptors are identified.

15 Kit for detection of cellular $FC_\epsilon R_{II}$

I) Detection of $FC_\epsilon R_{II}$ on peripheral blood cells.

Conventional IgE rosette assays suggest that atopic patients display increased expression of $FC_\epsilon R_{II}$ on
20 their blood mononuclear cells. Mab 25 permits a much more accurate determination of $FC_\epsilon R_{II}$ expression and is useful in this type of clinical investigation. Determining the expression of $FC_\epsilon R_{II}$ on blood mononuclear cells is useful in a number of clinical situations
25 related to atopy. The Mab 25 can be made available in a lyophilized form either unlabelled in itself but accompanied by an appropriately labelled second stage antimouse Ig antibody or labelled with either FITC or biotin. To detect biotin as label, FITC- or
30 phycoerythrin-labelled avidin can be used.

II) Detection of $FC_\epsilon R_{II}$ on human T cell clones.

It has been demonstrated in rodents that $FC_\epsilon R_{II}$ -positive T cells play a major role in the
35 regulation of IgE synthesis. Mab 25 is presently used to

establish human $FC_\epsilon R_{II}$ -positive IL_2 -dependent human T cell clones. Such clones should prove highly valuable tools to elucidate the regulation of IgE production in humans.

5

Kit for detection of soluble $FC_\epsilon R_{II}$ by competition assay

10 Mab 25 is coupled to beads or wells of microtiter plates. Samples to be assayed for the presence of soluble $FC_\epsilon R_{II}$ or a standard preparation of $FC_\epsilon R_{II}$ are first incubated with a known amount of labelled soluble $FC_\epsilon R_{II}$. After incubation they are
15 transferred to and allowed to react with the insolubilized antibody on the beads or in the wells. The labelled soluble $FC_\epsilon R_{II}$ can be either i) a ^{125}I iodinated recombinant $FC_\epsilon R_{II}$; ii) an artificial fusion product between $FC_\epsilon R_{II}$ and an enzyme e.g. E. coli β -
20 galactosidase; or iii) a biotinylated $FC_\epsilon R_{II}$. In case i) the reaction will be monitored through gamma counting; in case ii) the reaction will be monitored through optical measurement of degradation substrates specific for the enzyme (e.g. the reaction will be monitored by β
25 galactosidase); in case iii) optical measurement of degradation substrates specific for the enzyme which was coupled to the biotin-specific avidin. In any case the presence of soluble $FC_\epsilon R_{II}$ in the sample to be assayed will induce a decrease in the observed signal.

CLAIMS

1. Monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes.
- 5 2. Monoclonal antibodies to the human lymphocyte IgE receptors.
3. Antibodies according to claim 1 or claim 2 that are of immunoglobulin subclass IgG₁, in particular those designated Mab 25 and Mab 135 herein.
- 19 4. Monoclonal hybridomas capable of producing monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes.
- 15 5. Monoclonal hybridomas capable of producing monoclonal antibodies to the human lymphocyte IgE receptors.
- 20 6. The monoclonal hybridomas designated herein as 9P25 and 9P135 D6/5.
- 25 7. Use of a monoclonal antibody as claimed in any of claims 1 to 3 to assay cells bearing the human Fc_εR_{II} receptors.
8. Use of Mab 25 to immunoprecipitate a 42-44 Kd membrane molecule which is the Fc_εR_{II}.
- 30 9. Use of Mab 135 to immunoprecipitate three membrane molecules with molecular weights in the regions of 44-46, 35 and 30 Kd.

10. Use of Mab 25 in a competition immunoassay for the detection of soluble $\text{Fc}_\epsilon\text{R}_{\text{II}}$ and IgE BF.
 11. Use of Mab 25 to establish human IL_2 -dependent-T cell lines bearing the $\text{Fc}_\epsilon\text{R}_{\text{II}}$.
- 5



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12P 21/00, C07K 15/00
C12N 5/00, 15/00, G01N 33/577
// (C12P 21/00, C12R 1:91)

A3

(11) International Publication Number: WO 87/ 07302

(43) International Publication Date: 3 December 1987 (03.12.87)

(21) International Application Number: PCT/EP87/00264

(22) International Filing Date: 20 May 1987 (20.05.87)

(31) Priority Application Number: 86/07583

(32) Priority Date: 27 May 1986 (27.05.86)

(33) Priority Country: FR

(71) Applicant (for all designated States except US): LABOR-
ATOIRES UNICET [FR/FR]; 92, rue Baudin, F-
92307 Levallois-Perret (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BONNEFOY, Jean,
Yves [FR/FR]; Les Terrasses de Parsonges, Bâtiment
E, F-69570 Dardilly (FR). WIJDENES, John [NL/
FR]; 8, rue Jean Perréal, F-69008 Lyon (FR). PER-
ONNE, Catherine [FR/FR]; 70, rue de la Charité, F-
69002 Lyon (FR). AUBRY-LACHAINAYE, Jean-Pi-
erre [FR/FR]; 4, rue des Fraisières, F-69630 Chaponost
(FR). BANCHÉREAU, Jacques [FR/FR]; 25, avenue
Paul Santy, F-69130 Ecully (FR).

(74) Agent: DURAND, Yves; Cabinet Weinstein, 20, ave-
nue de Friedland, F-75008 Paris (FR).

(81) Designated States: AT (European patent), BE (Euro-
pean patent), CH (European patent), DE (European
patent), FR (European patent), GB (European pa-
tent), IT (European patent), JP, LU (European pa-
tent), NL (European patent), SE (European patent),
US.

Published

With international search report.

*Before the expiration of the time limit for amending the
claims and to be republished in the event of the receipt
of amendments.*

(88) Date of publication of the international search report:
14 January 1988 (14.01.88)

(54) Title: MONOCLONAL ANTIBODIES TO HUMAN LYMPHOCYTE IgE RECEPTORS, HYBRIDOMAS PRODUCING SUCH ANTIBODIES, AND KITS FOR USING SUCH ANTIBODIES

(57) Abstract

Monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes, to monoclonal antibodies to human lymphocyte IgE receptors, and to hybridomas producing such antibodies. These antibodies are preferably of the immunoglobulin subclass IgG₁. They can be used in assaying soluble Fc_εR_{II} and cells carrying these Fc_εR_{II} receptors. The invention further provides kits for assaying soluble Fc_εR_{II} and also cells carrying these Fc_εR_{II} receptors by means of monoclonal antibodies described herein.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 87/00264

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 P 21/00; C 07 K 15/00; C 12 N 5/00; C 12 N 15/00;
IPC: G 01 N 33/577; // (C 12 P 21/00; C 12 R 1:91)

II. FIELDS SEARCHED

Classification System ¹	Minimum Documentation Searched ⁷
IPC ⁴	Classification Symbols
	G 01 N
	C 12 P
	A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Chemical Abstracts, volume 104, no. 15, April 1986, (Columbus, Ohio, US), see page 556, abstract 128114y, & JP, A, 60255734 (NICHIREI CORP.) 17 December 1985	1, 2, 4, 7, 10
X	Biological Abstracts, volume 29, no. 17368, 1985, (Philadelphia, PA., US), T. Nakajima et al.: "Identification of the membrane molecules from RPMI-8866 cells reacting with amonoclonal anti- body specific to FC-epsilon-R", see the whole abstract & Journal of Allergy and Clinical Immunology(US) 1985, vol. 75, no. 1, part 2, p. 139	1, 2, 4, 7, 10
X	Biological Abstracts, volume 29, no. 15591, 1985, (Philadelphia, PA., US), M. Sarfatiet al.: "Production of anti- human FC-epsilon-R antibody upon immunization with monoclonal anti-	

* Special categories of cited documents: 14

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search
8th November 1987

Date of Mailing of this International Search Report

11 DEC 1987

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	human immunoglobulin E antibody", see the whole abstract & Federation Proceedings (US), 1985, vol. 44, no. 3, p. 784 --	1,2
X	Biological Abstracts, volume 80, no. 6, 1985, (Philadelphia, PA., US), T. Nagai et al.: "T and B lymphocytes with immunoglobulin E Fc receptors in patients with nonallergic hyperimmuno- globulinemia E: Demonstration using a monoclonal antibody against immuno- globulin E fc receptor-associated antigen", see page AB-456, abstract 49916, & Clin. Immunol. Immunopathol 35(3): 261-275, 1985 --	1,2,4,7, 10
A	The Journal of Immunology, volume 30, no. 4, April 1983, The American Association of Immunologists, (US), D.S Finbloom et al.: "Isolation of cross-linked IgE-receptor complexes from rat macrophages", pages 1489-1491 see the whole article --	1,8,9
X	The Journal of Immunology, volume 129, no. 2, August 1982, The American Association of Immunologists, (US), F.M. Melewicz et al.: "Comparison of the Fc receptors for IgE on human lymphocytes and monocytes", pages 563-569 see the whole article --	1,2,4,7, 10
A	The Journal of Immunology, volume 132, no. 2, February 1984, The American Association of Immunologists, (US), D.H. Conrad et al.: "The murine lympho- cyte receptor for IgE. I. Isolation and characterization of the murine B cell Fc γ receptor and comparison with Fc γ receptors from rat and human", pages 796-803 see abstract; introduction; discussion --	1,2,8,9
T	The Journal of Immunology, volume 137, no. 4, 15 August 1986, The American Association of Immunologists, (US), N. Noro et al.: "Monoclonal antibody (H107) inhibiting IgE binding to FC γ R (+) human lymphocytes", pages 1258-1263, see the whole article --	1-11
	./...	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
T	<p>The Journal of Immunology, volume 137, no. 4, 15 August 1986, The American Association of Immunologists, (US), M. Suemura et al.: "Monoclonal anti- Fcγ receptor antibodies with different specificities and studies on the ex- pression of Fcγ receptors on human B and T cells", pages 1214-1220 see the whole article</p> <p>-----</p>	1-11